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Clonality in Kaposi's Sarcoma

To the Editor: Rabkin et al. (April 3 issue)[1] studied multiple biopsy specimens from eight patients with Kaposi's sarcoma and claim that Kaposi's sarcoma begins as a clonal disease at some specific site, circulates in the blood, and implants itself at multiple sites in the skin to produce multicentric disease. The unbalanced methylation pattern at the androgen-receptor locus in Kaposi's sarcoma lesions provides the evidence for their view.

We are concerned about some of the authors' technical procedures. They do not provide results of amplification by the polymerase chain reaction with androgen-receptor primers and undigested DNA, to ensure that the observed pattern is truly due to allelic methylation. Moreover, the *Hpa*II restriction digestion was performed with heat-denatured samples, in which incorrect re-annealing can lose the original *Hpa*II site or generate additional sites. The use of proteinase inhibitors, such as phenyl methyl sulfonyl fluoride, after digestion with proteinase K can circumvent this problem. The authors do not give the age of the lesions before biopsy. Assuming that Kaposi's sarcoma begins as polyclonal disease that then evolves to a clonal disease, lesions that have been present for many months are more likely to have clonal patterns.

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We have studied tumor tissue from 11 women with Kaposi's sarcoma, all of whom were polymorphic for CAG repeats in exon 1 of the androgen receptor (Table 1). Tumor regions were microdissected and analyzed by a method similar to that of Rabkin et al.[1] In some cases more than one discrete tumor region from the biopsy specimen was assayed. Tumor-biopsy specimens from five of the women had evidence of clonality. Two women, from whom one and nine specimens were obtained, had evidence of more than one clone (Table 2). In two of the five women with clonal disease, there were areas of the tumor that were not clonal. We think that Kaposi's sarcoma is clonal in some cases but that independent clones

may develop, either spontaneously or after undergoing a premalignant hyperplastic stage. To investigate these possibilities, a large study is needed in which the age of cutaneous lesions is carefully recorded and specimens obtained from visceral tumors are included.

View this table: [in this window] [in a new window] **Table 1.** Methylation Patterns of the Tumor-Biopsy Specimens Studied.

View this table: [in this window] [in a new window] **Table 2.** Two Patients with Discordance of the Methylated Allele, Indicating the Presence of at Least Two Clones.

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1. Rabkin CS, Janz S, Lash A, et al. Monoclonal origin of multicentric Kaposi's sarcoma lesions. N Engl J Med 1997;336:988-993.[Abstract/Full Text]

To the Editor: Rabkin et al. readdress the topic of the clonal origin of neoplasms that frequently present as multiple lesions, such as Kaposi's sarcoma in patients with human immunodeficiency virus infection.[1,2] This issue has been raised before with regard to other synchronic tumors, such as transitional-cell neoplasms[3] and head and neck cancers.[4] Most of the information has been obtained by techniques that involve the random inactivation of one X chromosome in female subjects, as exemplified in the article by Rabkin et al.[1] This issue cannot be resolved in a straightforward fashion, especially by a technique in which each cell has only two possibilities for X-chromosome inactivation: inactivation of the X chromosome inherited from the father or of that inherited from the mother.

The authors used a statistical analysis that showed the low likelihood that their findings would occur by chance. Their assumption of concordant patterns of allele methylation is correct with respect to cell–cell comparisons, but their findings are based on DNA extracted from many cells after the microdissection of tumor nodules. Under these circumstances, each tissue sample from a female subject with informative alleles can be polyclonal, monoclonal with preferential methylation of the larger allele, or monoclonal with the smaller allele predominating. Assuming that there is an equal probability of each of these three

patterns, the likelihood of a concordant pattern would be $2/3^n$ (where 2 is the number of alleles and n the number of tumors being compared); that is, $2/3^2$ (or approximately 22 percent) for two tumors, $2/3^3$ (approximately 7.4 percent) for three tumors, $2/3^4$ (approximately 2.5 percent) for four tumors, and $2/3^5$ (approximately 0.8 percent) for five tumors. Under these circumstances, the probability that different tumors from the same patient have a monoclonal origin is greater than the authors calculate.

We suggest caution in the interpretation of these calculations because of the skewing of X-chromosome methylation in different tissues. Synchronic or metachronic tumors can be shown to be truly monoclonal (derived from the same clone) only if several molecular markers are concordant. An analysis of X-chromosome inactivation cannot distinguish metastatic tumors (arising from a single clone) from synchronic tumors arising from different clones but expressing the same inactivated X chromosome. The results obtained by analyzing a single molecular marker prove clonal expansion of a subgroup of tumor cells with proliferative advantages, but they do not prove actual clonality, especially when the assay is based on patterns of methylation that may be influenced by the functional status of the cell, tumor progression, or both.[5]

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The authors reply:

To the Editor: Gill et al. report balanced methylation patterns in 26 of 40 microdissected specimens of Kaposi's sarcoma (65 percent). They correctly note that balanced methylation could reflect mixed clonality of tumor or stromal cells. However, only 4 of the 32 Kaposi's sarcoma tumors we studied (12 percent) had balanced methylation. Although the difference could be due to variation in the frequency of polyclonal disease, it could also be explained by varying degrees of success in obtaining sufficiently pure preparations of tumor cells.

The two patients described by Gill et al. who had discordant methylated alleles could represent cases in which there are multiple primary lesions, but we found no discordance in a larger number of clonal samples. The technical issues they raise do not explain the discrepancy. We observed balanced *HUMARA* amplification with undigested tumor DNA and balanced *Hpa*II restriction after inactivation of normal-skin DNA by heat. If multicentric Kaposi's sarcoma lesions evolve independently, we should also have seen cases in which there was discordance of the methylated allele. Our model of a circulating neoplastic progenitor is suggested by the statistical improbability that our results occurred by chance alone.

Drs. Diaz-Cano and Wolfe correctly note that Kaposi's sarcoma tissue from a female subject with informative alleles can reveal either a polyclonal or a monoclonal pattern of *HUMARA* allele methylation. However, we had no basis for assigning relative probabilities to these alternatives in our statistical analysis. Instead, we calculated the conditional probability of concordant allelic methylation given a monoclonal pattern. We disagree with the writers' interpretation that polyclonal and monoclonal patterns in different tumors indicate that the tumors have different clonal origins, since an admixture of stromal and tumor DNA may appear to be polyclonal. Nevertheless, our Patients 1, 4, and 5 each had monoclonal patterns in all the tumors that could be evaluated. Under the assumptions proposed by Drs. Diaz-Cano and Wolfe, the combined probability that this result would occur by chance is less than 0.00001 ($2/3^5 \times 2/3^5 \times 2/3^3$). Thus, even their assumptions lead to a conclusion of monoclonality, at least in some patients.

We appreciate the potential problem of skewed methylation of normal tissue precursors.[1] Normal skin from six of the eight patients we studied had balanced methylation patterns, but it is uncertain what is the most appropriate normal control for Kaposi's sarcoma. However, changes in DNA methylation that occur during tumorigenesis [2] cannot explain skewing of X-chromosome methylation in the absence of either clonal expansion or X-linked differences in the cellular propensity to Kaposi's sarcoma (for which there is no other evidence). We agree that detecting additional genetic changes would support the evidence of clonality provided by the *HUMARA* assay, which has contributed to the current understanding of histiocytosis X,[3] desmoid fibromatosis,[4] and Rosai–Dorfman disease.[5]

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